Oral Session 4

Identification of *Bacillus* spp., *Escherichia coli*., *Salmonella* spp., *Staphylococcus* spp. and *Vibrio* spp. with 16S Ribosomal DNA Based Oligonucleotide Array Hybridization

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ABSTRACT

Rapid identification of the genus and species of bacteria in foods and clinical specimens is important. In this report, DNA sequences of bacterial 16S rDNA were used to develop the oligonucleotide array for the identification of bacterial strains of *Bacillus* spp., *Escherichia coli*, *Salmonella* spp., *Staphylococcus* spp. and *Vibrio* spp. Most of these bacterial strains may cause food-borne outbreaks or sporadic cases. A rapid (<4 h) detection method that used universal PCR primers to amplify the variable regions of bacterial 16S rDNA, followed by reverse hybridization of the PCR products, which were biotin labeled, to the oligonucleotides arrayed on the chip was developed. Fifteen oligonucleotide probes were selected and spotted on the nylon strip to determine the array hybridization patterns. It was successful in discriminating *Bacillus* spp., *Escherichia coli*, *Salmonella* spp., *Staphylococcus* spp. and *Vibrio* spp. with identification, in general, to the genus level, not species level. As 182 randomly selected strains were assayed, the detection rate was found higher than 98%. Except for 3 strains, the remaining 179 strains were correctly identified and no cross reactions were observed. These 179 strains generated five hybridization patterns. Adding more oligonucleotide probes to the array may allow the detection of more bacterial genera and species without significant increasing the complexity or cost.

Key words: 16S ribosomal DNA, Oligonucleotide array, Food pathogen.

INTRODUCTION

Bacterial identification methods currently used are based on the analysis of morphological, physiological, biochemical, and genetic data of the target bacteria. In the last decade, rapid diagnostic methods have been developed by direct detection of the specific genes of bacteria in clinical and food samples, and many species or genus specific oligonucleotides have been developed for such detection (Laberge *et al.*, 1996; Newcombe *et al.*, 1996; Hsu and Tsen., 2001; Chizhikov *et al.*, 2001; Tsen and Chen., 2001; Rudi *et al.*, 2000). These systems are usually used to confirm the diagnosis of specific clinical syndromes or specific bacterial strains (Chun *et al.*, 1999; Daffonchio *et al.*, 1998; Tsen *et al.*, 1999). These results suggest that DNA based methods may be more sensitive and rapid than the conventional bacteriological methods.

However, the use of different primers for various bacterial strains is impractical for the routine analysis of food or clinical samples that may contain one or more of many possible pathogens. For such specimens, either a complex PCR with a mixture of large numbers of primers is needed, or a large series of individual PCRs must be run in parallel, or sequentially. Multiplex PCR may be effective for a limited number of organisms, but as more primers are added, the detection sensitivity for some target bacteria decreases and the chance that two unrelated primers produce spurious product increases (Tsen *et al.*, 2001). These problems can be solved by using a biochip arrayed with oligonucleotides specific for different bacteria species.

Recently, 16S rRNA gene (*rrn*) operon has been used for the designing of DNA probes and PCR primers specific for some bacteria genera and well-solved species, such as *E. coli* (Tsen *et al.*, 1998), *Listeria monocytogenes*, *Salmonella* spp. (Gendel *et al.*, 1996; Lin *et al.*, 1996; Tsen *et al.*, 1999), and *Bacillus cereus* group species, including *B. cereus*, *B. thuringiensis*, *B. anthracis*, and *B. mycoides* (Ash *et al.*, 1991; Giffel et al., 1997). Genetic variations in 16S rDNA are intergeneus and interspecies for some well studied species and this variation could be utilized for the designing of oligonucleotides used in array identification system. Bacteria could be identified directly by amplification of bacterial 16S rDNA, 23S rDNA or the intergenic spacer region (ISRs), followed by reverse hybridization of the labeled amplified product to an oligonucleotide array designed to differentiate the sequence variation of the bacteria. With such a principle, Anthony et al. (2000) has developed a 23S rDNA based oligonucleotide array system for the rapid diagnosis of bacteremia. Also, a very recent report showed that using 16S rDNA PCR and DNA array, 15 fish pathogens might be simultaneously discriminated (Warsen *et al.*, 2004).

In this report, by using an array of specific oligonucleotides designed from the variable regions of 16S rDNA sequences, and the reverse hybridization of 16S rDNA amplified products to this array, we established the specific hybridization patterns for strains of *Bacillus* spp., *Escherichia coli*, *Salmonella* spp., *Staphylococcus* spp., and *Vibrio* spp.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains used in this study are listed in Table 1. These strains were obtained from American Type Culture Collection (ATCC, Rockville, MD, U.S.A.); Culture Collection and Research Center (CCRC, Hsin-Chu, Taiwan); National Pingtung University (PT, Pingtung, Taiwan); United State Department of Agriculture (USDA, Washington D.C., USA) and Taiwan Agriculture Chemical and Toxic substances Research Institute (TACTRI, Taichung, Taiwan).

Table 1. Bacterial strains and their oligonucleotide array hybridization patterns.

Strains	Pattern	No. of strains	Strain no. and sources
B. cereus group	C	23	*B. cereus* ATCC 14579, 13061, 21182, 21281; CCRC 10250, 10282, 10603; Bc16-31 (this lab).
		30	*B. thuringiensis* ATCC 33679, 10729; CCRC 11501, 14377, 15070;Tt01~25 (TACTRI).
		3	*B. mycoides* ATCC 6462, 10206; CCRC11968.
		2	*B. anthracis* ATCC 8705, 14578.
Other *Bacillus* spp.	C	9	*B. subtilis* (CCRC 10029, 10258, 10267, 10447); *B. polymyxa* (ATCC 842); *B. coagulans* (CCRC 10251); *B. firmus* (ATCC 14575); *B. pumilus* (ATCC7061); *B. lentus* (ATCC10804).
E. coli	A	25	ATCC 11775, 25922, 43886 (ETEC), 43894 (EHEC); CCRC 12570, 14824 (EHEC), 13086 (EHEC), 41443; WHO 110 (ETEC); E01~08; E2418~2425 (this lab).
Salmonella spp.	B	5	*S. enterotidis* ATCC 13076; PT 633,661; SE06, 07 (this lab).
		7	*S. typhi* CCRC 14875; ATCC 8427; ST01-04, 40 (this lab).
		10	*S. typhimurium* ATCC 14028, 13311; STM01-03, ST604, ST62, 63, 80, 90 (this lab).
		32	*S. aba; S. anatum; S. arkansas; S. azteca; S. bareilly; S. berta; S. borismorbificans; S. braenderup; S. choleraesuis; S. cerro; S. coleypark; S. colindale; S. derby; S. dublin; S. emek; S. eppendrof; S. florida; S. goerlitz; S. hadar; S. havana; S. infantis; S. meleagridis; S. memhaden; S. montevideo; S. muenchen; S. newport; S. oranienburg; S. panama; S. richmond; S. saintpaul; S. victoria; S. worthington.*
Staphylococcus spp.	D	10	*Staph. aureus* CCRC 12653, 12654, 12656, 12657, 12660, 13831; STA 0029, 0040, 0042, 0043 (this lab.).
		5	*Staphy. epidermidis* (ATCC 155); *Staphy. haemolyticus* (ATCC 29970); *Staphy. hominis* (ATCC 27844); *Staphy. simulans* (ATCC 11631); *Staphy. warneri* (ATCC 27836).
Vibrio spp.	E	10	*V. parahaemolyticus* CCRC 12863, 12963, 13023, 13025; VP 1253, 1273, 1287, 1311, 1328, 1354 (this lab.).
		11	*V. alginolyticus; V. cholerae; V. cincinnatiensis; V. fluvialis; V. furnissii; V. harveyi; V. mediterranei; V. mimicus; V. proteolyticus; V. tubiashii; V. vulnificus.*
Other bacteria spp.		4	*Pseudomonas aeruginosa* (ATCC 10145), *Latobacillus brevis* (CCRC 12187), *Proteus vulgaris* (CCRC 10728), *Streptococcus faecalis* (CCRC 10066)

PCR primers for 16S rDNA

The primer sequences were chosen from the conserved regions previously reported for the bacterial 16S rDNA. Partial 16S rDNA sequences flanking variable regions (V) from V1 to V3 were used. The PCR primers were designed as follows: forward primer, 16S-350F and reverse primer, 16S-820R. Primers were synthesized with a model 394 DNA-RNA synthesizer (Applied Biosystems, Foster City, Calif.).

DNA isolation and PCR amplification

Genomic DNA was extracted and purified using Genomic Tips (Qiagen, Valencia, Calif.) with Genomic DNA Buffer Set (Qiagen). The concentration of DNA was determined by UV spectrophotometry and with SpotCheck (Sigma, St. Louis, Mo.). 16S rDNA fragment was amplified by using the primer set 16S-350F and 16S-820R that were targeted to universally conserved regions. The amplification reactions were performed with a GeneAmp 2400 PCR thermocycler (Perkin-Elmer, Norwalk, Conn.). The PCR products were confirmed by 2% agarose gel electrophoresis. For biotin labeled PCR product preparation, similar conditions were used except that the primers used were 5'-biotin labeled 16S-350F and 16S-820R. These biotin primers were synthesized by MDBio Inc. (Taipei, Taiwan).

Oligonucleotide synthesis and array fabrication

BLAST search was used to find and retrieve the sequences homology for each 16S rRNA genes. Included in the procedure were 16S rDNA sequences (with accession number) of *Salmonella enteritidis* (U90318), *Vibrio parahaemolyticus* (AB016271), *Salmonella typhi* (U88545), *Escherichia coli* (J01859), *Salmonella typhimurium* (U90316, U88546), *Staphylococcus aureus* (AF146368), and *Bacillus cereus* (Z84576). Using the CLUSTAL X Multiple Sequence Alignment Program (Strasburg, France), nucleotide sequences of the bacterial 16S rDNA obtained from the Gene Bank database were aligned and the oligonucleotides (Table 2) could be designed using the ArrayDesigner software (PREMIER Biosoft, CA, USA.). Oligonucleotides purified from C18 Nucleosil column (Sigma, St. Louis, Mo.) were melted in TE buffer and spotted onto the nylon membrane (Hybond-N+, Amersham, USA.) by using the Manual Glass Slide Indexing System (VP 470 and 478A, V&P SCIENTIFIC Inc., CA, USA.). Each spot containing 5 ng oligonucleotide DNA. The spotted DNA was then cross linked onto to the nylon membranes by UV irradiation with CROSSLINKER CL-508 (UVItec, Cambridge, ENGLAND) at 0.12 joule/cm^2. The membranes were stored at 4 ℃ before use.

Hybridization and chromogenic reaction

The biotin-labeled 16S rDNA PCR amplicons were hybridized to the oligonucleotides arrays using the following protocol. Each membrane strip was numbered and placed in a separate 1.5-ml screw-top microcentrifuge tube containing 1.0 ml of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% N-laurylsarcosine, 0.05% SDS, 10 μg of heterologous salmon sperm DNA (Sigma Chemical Co.), and 1% blocking reagent (Roche, IN., USA). The biotin labeled PCR products were heated to 99℃ for 3 min and cooled to 4 ℃ immediately in the PCR thermal cycler, followed by transferring to

each proper hybridization tube. Hybridization was carried out for 60 min at 50℃ with gentle agitation.The membrane strips were removed from the tubes and washed twice with 5 ml 0.1× SSC-0.1% SDS at 37℃ for 5 min. They were then blocked with 2 ml of blocking reagent (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.5% skim milk) for 15 min followed by incubation for 30 min with the same buffer containing 1/1000 volume of streptavidin-alkaline phosphatase (NEN, Boston, MA, USA.). Afterwards, the strips were washed twice in 5 ml Tris-buffered saline (TBS, pH 7.4) followed by chromogenic reaction. For this reaction, the membrane strips were immersed into 1 ml 5-bromo-4-chloro -3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT) substrate solution (NEN, Boston, MA, USA.) with gentle agitation, and the color developed could be clearly observed after 10 to 30 min. After drying the strips at 65℃ for 5 min, the relative signal intensities were determined by scanning the membranes with an UMAX Power Look 3000 (UMAX, Texas, USA.). The results were analyzed by using the Gel-Pro ANALYZER software (Media Cybernetics, Silver Spring, Md. USA).

RESULTS

Design of PCR primers and fabrication of oligonucleotide array

To construct the oligonucleotide array for the detection of food-borne pathogen, the conserved regions flanking variable region 1 (V1) to 3 (V3) of the bacterial 16S rDNA (Fig. 1) were used to design the two consensus primers, 16S-350F and 16S-820R. Using the 5′ end biotin-labeled primers from 16S-350F and 16S-820R, DNA sequence flanking the V1 to V3 regions of the 16S rRNA genes for the bacterial strains to be detected could be amplified. Molecular weights of these PCR products were equal to 475 bp (Fig. 2). Target bacterial strains selected included strains of common food pathogens that may cause diarrhea or vomiting, strains of *B. thuringiensis*, which may produce enterotoxigenic activity (Yang *et al.*, 2003), and other bacterial strains (Table 1). Under the PCR conditions described in Methods, only very few strains, i.e., 3 of the total 182 strains assayed, could not generate any amplification products. For example, three strains, i.e., *Bacillus spirit*, *B. narit* and *Salmonella* Vento, gave negative PCR results. Lowering the stringent conditions may enhance the PCR for these strains.The oligonucleotides probes to be arrayed were obtained by use of the software Array-Designer (CA, USA.) (Table 2). All the bacterial 16S rDNA sequences available in NCBI Gene Bank database, were collected. Initially, 36 oligonucleotides were selected. PCR amplicons obtained from amplification of the bacteria chromosomal DNAs using consensus primers were then hybridized to the array. Most of the hybridization results were in agreement with those predicted from

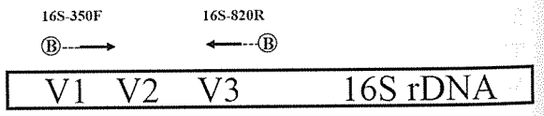

Fig. 1. Relative positions of the universal PCR primers. Both primers were labeled with biotin at the 5′end and after the PCR product was denatured, it served as probes to hybridize with the oligonucleotides arrayed on the nylon membrane.

Table 2. Oligonucleotide probes and PCR primers used in this work.

Oligonucleotides (dots) [a]	Sources[b]	Accession Number
No. 1	*B. cereus* (anti-sense)	Z84576
No. 2	*B. cereus* (anti-sense)	Z84576
No. 3	*B. cereus*	Z84576
No. 4	*B. cereus* (anti-sense)	Z84576
No. 5	*B. cereus*	Z84576
No. 6	*Staph. aureus*	AF146368
No. 7	*B. cereus (anti-sense)*	Z84576
No. 8	*E. coli*	J01859
No. 9	*B. cereus*	Z84576
No. 10	*Staph. aureus*	AF146368
No. 11	*E. coli*	J01859
No. 12	*V. parahaemolyticus*	AB016271
No. 13 [c]	*E. coli (forward primer)*	J01859
No. 14	*E. coli*	J01859
No. 15	*E. coli*	J01859
No. 16	*Salm. typhimurium*	U90316, U88546
16S-820R [c]	*E. coli (reverse primer)*	J01859

[a] Oligonucleotides No.1-16 were spotted onto the nylon membranes.

[b] 16S rDNA sequence from the bacteria shown was aligned with those of other bacteria and thus the oligonucleotides were designed.

[c] The universal forward (16S-350F) and reverse (16S-820R) primers were biotin labeled on the 5′ end.

the probe sequences. Each PCR product hybridized to its corresponding specific oligonucleotide probe, but some PCR amplicons cross hybridized with other tested oligonucleotide probes. For example, PCR amplicons generated from *Salmonella* spp. hybridized with No. 15 oligonucleotide probe which was deduced from *E. coli* 16S rDNA. Also, PCR amplicons from *Vibrio* spp. hybridized not only with its own specific oligonucleotide No. 12, but also with oligonucleotide probes No. 8 and 15. Probes No. 8 and 15 were both deduced from the *E. coli* 16S rDNA (Table 2). Some of the designed oligonucleotide probes were eliminated from the membrane strip, unless they were useful for the pattern differentiation. Also, oligonucleotide probes that could not hybridize with any bacterial PCR amplicons were excluded from the array. After spotting the useful fifteen oligonucleotide probes on the membrane strip followed by hybridization with biotin labeled PCR products prepared from bacterial strains shown in Table 1, five hybridization patterns (A → E) (Fig. 3) corresponding to five bacteria genera, i.e., *E. coli*, *Salmonella* spp., *Bacillus* spp., *Staphylococcus* spp. and *Vibrio* spp., (Table 1, Fig. 3) were obtained.Due to the fact that oligonucleotide probe No. 11 could hybridize with amplicons from all the 179 bacterial strains tested plus some other unlisted strains (data not shown), it was thus used as the probe for positive control. Also, probe No. 13, equivalent to biotin-labeled primer 16S-350F, serve as a positive control for the chromogenic reaction.

Different parameters for array hybridization, such as temperature, incubation time, salt composition and shaking speed, were optimized for both the specificity and sensitivity of the array system. The optimized conditions were given in the experimental methods. For hybridization patterns, all the *E. coli* strains tested were able to hybridize with probes No. 8, 11, 14 and 15 and generate a specific pattern A. From the patterns shown in Fig. 3, it may be concluded that oligonucleotide No. 14 is a species specific probe for *E. coli*, although strains of the closely related genus *Shigella* also generate the same pattern (data not shown). Also, oligonucleotide No. 16 and No. 12 are the genus specific probes for *Salmonella* spp. and *Vibrio* spp., respectively. *Salmonella* and *Vibrio* spp. generated a specific oligonucleotide array hybridization patterns B and E, respectively (Fig. 3). Meanwhile, probes No. 4, 5, 6, 7, 9, 10 and 11 were the co-shared probes for strains of *Bacillus* spp. and *Staphylococcus* spp., and probes No. 1-3 were genus specific probes for strains of *Bacillus* spp. Almost all the *Bacillus* strains tested produced the specific hybridization pattern C. For *Staphylococcus* spp., all the *Staphylococcus* strains tested generated hybridization pattern D, from which, however, specific and unique probes for *Staphylococcus* spp. could not be found.

In order to confirm the hybridization patterns shown in Fig. 3, all the 182 isolates belonging to the five genera were analyzed and 179 strains generated the expected hybridization patterns (Table 1). The 3 strains showing negative hybridization results could not generate any PCR amplicon with the consensus primers.

Sensitivity and specificity

Under the experimental conditions, PCR products amplified from 1~5 X 10^3 cfu/ml of the cell dilutions could be observed when the products were electrophoresed with 2% agarose gel (Fig. 2). In some cases, the PCR product was not visualized on the ethidium bromide stained agarose gel, however, after hybridization of the PCR products to the oligonucleotide array, hybridization signals were visualized. Thus, PCR method followed by hybridization of the products to the oligonucleotide array would improve the detection sensitivity. For the bacterial strains tested, the detection limit for *E. coli* and *V. parahaemolyticus* (500 cfu / ml) was the lowest, followed by *Salmonella* spp. On the other hand, *Staphylococcus* spp. and *Bacillus* spp., gave higher detection limits, i.e., about 4,500 cfu/ml. Chromosomal DNA from *Pseudomonas aeruginosa* (ATCC 10145), *Latobacillus brevis* (CCRC 12187), *Proteus vulgaris* (CCRC 10728), *Streptococcus faecalis* (CCRC 10066) could produce proper PCR amplicons with the consensus primers but not the visible array hybridization signals.

To further confirm the identification results, some genus specific oligonucleotide probes were selected and arrayed in three repeats onto new strips (Fig. 4). For such analysis, all the target strains could produce expected hybridization patterns. For example, all the 58 *Bacillus* strains assayed hybridized with oligonucleotide No. 1 and No. 2, and produced pattern "a" in Fig. 4. All the strains of other bacteria spp; i.e., *E. coli*, *Salmonella* spp. and *Vibrio* spp. produced their own specific hybridization patterns, i.e., pattern b, c, d, respectively, on such new arrayed strips (Fig. 4). These results demonstrate that these five selected oligonucleotide probes could serve as genus specific probes.

DISCUSSION

For simultaneous detection of two or more bacterial pathogens, multiplex PCR with several sets of species-specific primers could be used (Tsen *et al.*, 1999; Lin *et al.*, 1996). However, in some cases, different primer sets may generate PCR products with molecular weights similar or closer to each other. Also, mixture of several primer sets might lead to poor amplification efficiency, and lowering the annealing temperature might generate false-positive results. Modification of the multiplex PCR conditions may not be able to eliminate the interference caused by false-positive bands (Wilson *et al.*, 2002). The method described in the present study resolves these problems. Only a single running of PCR with a single set of primers derived from the conserved sequences of a common gene is needed.

The common gene used for the designing of oligonucleotide array is 16S rDNA since it is generally accepted that the sequence of 16S rRNA can be used to distinguish genera and well resolved species (Plays *et al.*, 1997). However, the rRNA (*rrn*) operons are often multigenic in bacteria genome with the number of *rrn* operons ranging from 1 to 15 (Acinas *et al.*, 2004). For example, both *Salmonella* spp. and *E. coli* contain seven *rrn* operons (Condon *et al.*, 1995). Thus, oligonucleotide probes or primers designed from the bacterial 16S rDNA sequence retrieved from the database in Genebank may not allow the detection of all strains in a bacteria species (Lin *et al.*, 2004). It should be mentioned that sequence variation between 16S rDNA copies is usually < 1%, however, this may be greater between strains of the same species depending on the region being used as a probe.

It is sometimes difficult to find suitable signature sites on the 16S rRNA for the identification of bacteria on the species level.

In this regard, the 23S rRNA with approximately 3,000 nucleotides has twice as many potential probe target sites as the 16S rRNA (Fuchs *et al.*, 2001). However, there are not many 23S rRNA sequences in the databases, compared to more than 18,000 16S rRNA sequences (Maidak *et al.*, 2000). Also, by the use of longer probes, it is consequently not helpful for fast and unambiguous identification of closely related species in the DNA array hybridization reaction (Hedegaard *et al.*, 1999). Using the

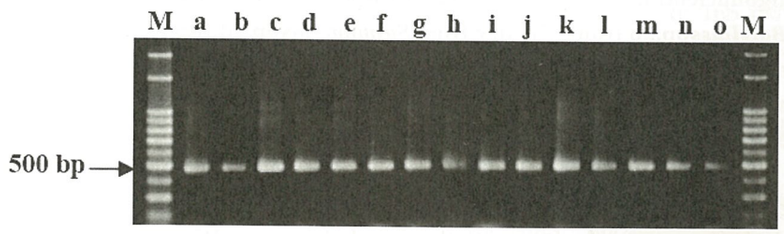

Fig. 2. PCR products amplified with the universal 16S rDNAPCR primers. PCR products amplified from different strains, i.e., lanes: a-e, *E. coli*ATCC 11775, 25922, 43894, 43894, CCRC 12570; f-j, *B. thuringiensis*ATCC 33679, 10729, CCRC 11501, 14377, 15070; k-o, *S. aureus*CCRC 12653, 12654, 12656, 12657 and 12660, are shown. Lane M represent the 100-bp DNA ladder.

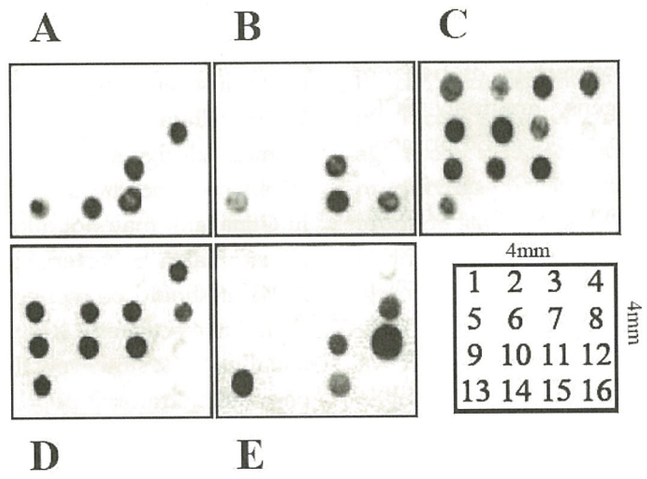

Fig. 3. Bacteria hybridization patterns using the 16S rDNAoligonucleotide array. The patterns are Afrom *Escherichia coli*; Bfrom *Salmonella*spp.; Cfrom *Bacillus*spp.; Dfrom *Staphylococcus* spp.and Efrom *Vibrio* spp. The numbers of the oligonucleotides (Table 1.) spotted on the membrane were indicated in the lower right-hand strip. Dot No. 13 was the biotin labeled forward primer which served as the chromogen development positive control.

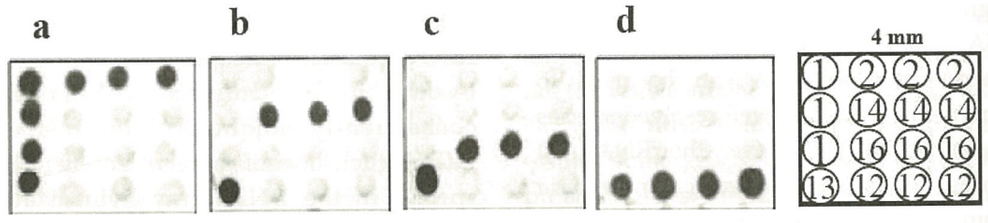

Fig. 4. The oligonucleotide three repeats verification. The hybridization patterns obtained are a from *Bacillus*spp.; b from *E. coli*; c from *Salmonella*spp.; d from *Vibrio*spp. The numbers of oligonucleotides spotted on the membrane were indicated on the strip. These oligonucleotides were those of No.1, 2, 12, 14 and 16, corresponded to those in Table 2. Dot No. 13 served as a chromogenic positive control.

23S rDNA based array system, when 158 detected, only 125 (79.7%) were correctly identified (Anthony *et al.*, 2000). Although a 16S rDNA based microarray has been developed for the discrimination of 15 fish pathogens, for each bacteria species, only one strain was tested (Warsen *et al.*, 2004). In unselected positive blood culture broths was their report, Warsen *et al* (2004) pointed out that it is possible that a strain that should be identified using such microarray might be missed due to a random mutation within this region and thus produced a false negative. They do not know what the frequency of

these events might be at a population level. As described earlier, the rRNA gene are multigenic and the rDNA sequences they retrieved form the Genebank for oligonucleotides designing may represent only one rrn operon, which may not exist in some strains of the same species. Warsen *et al* (2004) also pointed out that an additional challenge with their strategy is that two different species may share the same sequence identity within the probe region (Fox *et al.*, 1992). In fact, 16S rRNA sequence identity may not be sufficient to guarantee species identity (Fox *et al.*, 1992, Plays *et al.*, 1997). Although the microarray shown in this report allows the discrimination of bacterial strains mainly on the genus level, the detection rate for bacterial strains was higher than 98.3%. This was shown by the assay of 182 strains shown in Table 1.

An ideal oligonucleotide should hybridize only to one bacterial species and to all members of the strains within that species. However, in this study, we found that probes sequences with homology higher than 85% to bacteria other than target strain may cross react with strains of these closely related species. In such cases, two or more additional oligonucleotides may be required to allow the discrimination of the bacterial strains (Fig. 3, pattern C, D). Using the array system, the clonal relationship between bacterial strains might be elucidated. For example, *Salmonella* spp. and *Vibrio* spp. were found to hybridize with oligonucleotide No. 15 which was obtained from *E. coli* 16S rDNA sequence (92% homology). It means that the phylogenetic distance of these three bacteria genera was much closer than that of the others. Thus, it is necessary to add one or more additional oligonucleotides to the array, depending on the clonal relationship, to allow the discrimination of these bacterial strains.

The variation in the intensity of the hybridization signal may be due to many factors including the extent of sequence homology; the variations of the probe concentrations and the differences in the hybridization stringency for different probes. In addition, cellular protein, lipid, and carbohydrate may mediate significantly nonspecific binding of biotin-labeled probes or variant isolates.

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